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Epigenetics and Early Life Stress: Experimental Brood Size Affects DNA Methylation in Great Tits (*Parus major*)

Bernice Sepers^{1,2*}, Jolijn A. M. Erven^{1,2}, Fleur Gawehns³, Veronika N. Laine^{1,4} and Kees van Oers^{1,2}

¹ Department of Animal Ecology, Netherlands Institute of Ecology (NIOO-KNAW), Wageningen, Netherlands, ² Behavioural Ecology Group, Wageningen University & Research (WUR), Wageningen, Netherlands, ³ Bioinformatics Unit, Netherlands Institute of Ecology (NIOO-KNAW), Wageningen, Netherlands, ⁴ Finnish Museum of Natural History, University of Helsinki, Helsinki, Finland

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and Food Research Ltd.,
New Zealand

*Correspondence:

Bernice Sepers
b.sepers@nioo.knaw.nl;
k.vanoers@nioo.knaw.nl

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Early developmental conditions are known to have life-long effects on an individual's behavior, physiology and fitness. In altricial birds, a majority of these conditions, such as the number of siblings and the amount of food provisioned, are controlled by the parents. This opens up the potential for parents to adjust the behavior and physiology of their offspring according to local post-natal circumstances. However, the mechanisms underlying such intergenerational regulation remain largely unknown. A mechanism often proposed to possibly explain how parental effects mediate consistent phenotypic change is DNA methylation. To investigate whether early life effects on offspring phenotypes are mediated by DNA methylation, we cross-fostered great tit (*Parus major*) nestlings and manipulated their brood size in a natural study population. We assessed genome-wide DNA methylation levels of CpG sites in erythrocyte DNA, using Reduced Representation Bisulfite Sequencing (RRBS). By comparing DNA methylation levels between biological siblings raised in enlarged and reduced broods and between biological siblings of control broods, we assessed which CpG sites were differentially methylated due to brood size. We found 32 differentially methylated sites (DMS) between siblings from enlarged and reduced broods, a larger number than in the comparison between siblings from control broods. A considerable number of these DMS were located in or near genes involved in development, growth, metabolism, behavior and cognition. Since the biological functions of these genes line up with previously found effects of brood size and food availability, it is likely that the nestlings in the enlarged broods suffered from nutritional stress. We therefore conclude that early life stress might directly affect epigenetic regulation of genes related to early life conditions. Future studies should link such experimentally induced DNA methylation changes to expression of phenotypic traits and assess whether these effects affect parental fitness to determine if such changes are also adaptive.

Keywords: parental effects, development, postnatal conditions, growth, physiology, behavior

INTRODUCTION

Developmental phenotypic plasticity can be defined as irreversible changes in the phenotype resulting from environmentally introduced alterations in development (Forsman, 2015). These changes can occur through parental effects, which occur when the parental environment or phenotype affects that of their offspring. Parents with extended brood care are known to affect their offspring via both prenatal and postnatal effects. Well known prenatal effects are those that occurred in humans after prenatal exposure to the Dutch famine, leading to for example lower glucose tolerance (de Rooij et al., 2006), obesity (Roseboom et al., 2006), diabetes (Kahn et al., 2009) and impaired selective attention (de Rooij et al., 2010). A classic example of a postnatal parental effect is that of maternal nursing and grooming on anxiety and stress response of rat pups (Weaver et al., 2004). A likely reason why parents adjust their offspring's phenotype is to maximize parental fitness (Reddon, 2012), by transferring information about the current environment to their offspring and subsequently shape their offspring's phenotype to match the environmental conditions. If these conditions remain stable, this might increase their offspring's reproduction or survival (Champagne et al., 2003; Dantzer et al., 2013).

Parents can passively pass on information about current environmental conditions via prenatal hormone secretion (Dloniak et al., 2006; Dantzer et al., 2013) and resource allocation (de Rooij et al., 2006; Roseboom et al., 2006; Kahn et al., 2009) as has been observed in mammals or by yolk hormone deposition as observed in various bird species (Schwabl, 1993; Bentz et al., 2016). For example, yolk testosterone in wild Eastern Bluebirds (*Sialia sialis*) is positively correlated with breeding density and nestling growth (Bentz et al., 2016). Parents can also transfer information about current environmental conditions in an active way, by for example grooming behavior (Champagne et al., 2003; Weaver et al., 2004), thereby shaping early environmental conditions of the offspring after birth. Such early developmental conditions provided by the parents are known to have long-term influences on their offspring's behavior (Carere et al., 2005; van Oers et al., 2015), physiology (Keller and van Noordwijk, 1994; DeKogel, 1997; Naef-Daenzer and Keller, 1999) and may also have fitness consequences (DeKogel, 1997; Naguib et al., 2006). Intergenerational parental effects indicate an information transfer from parent to offspring (Jablonka and Raz, 2009; Bošković and Rando, 2018) but this does not imply that the patterns will be stably inherited via parental germ cells (Heard and Martienssen, 2014; Guerrero-Bosagna et al., 2018).

Epigenetic mechanisms have repeatedly been suggested to mediate the parental regulation of offspring phenotype (Kappeler and Meaney, 2010; Groothuis and Trillmich, 2011; Kilvitis et al., 2014). These biochemical mechanisms stably alter gene expression by affecting either transcription or translation without a change in the primary nucleotide sequence of the genome. Since epigenetic mechanisms can be induced in response to the local environment (Weaver et al., 2004; Pertille et al., 2017; Zimmer et al., 2017; Liu et al., 2018) they are good candidates

to facilitate early developmental effects on offspring phenotype. The best-studied epigenetic mark is DNA methylation, which is the addition of a methyl group to a nucleotide. In vertebrates, this nucleotide is usually a cytosine in a CpG context, which is a CG dinucleotide (5'-cytosine guanine-3'), separated by a phosphate (p) group. Methylation can affect gene expression by interfering with the binding of proteins necessary for transcription initiation (Bird, 2002; Moore et al., 2013). Pre- and postnatal parental effects on offspring DNA methylation have been found in vertebrates like humans (Tobi et al., 2009, 2014), fish (McGhee and Bell, 2014), rats (Weaver et al., 2004) and mice (St-Cyr and McGowan, 2015), but this is hardly studied in altricial birds, even though there are some very suitable model systems.

In altricial birds in natural conditions, a major part of the early developmental conditions are largely determined by the parents, because the nestlings completely rely on their parents for nutrition. The parents are able to affect the quality and quantity of food per nestling by adjusting the egg laying date and brood size (Pettifor et al., 1988, 2001; Perrins and McCleery, 1989), prey choice, food selectivity (Wright et al., 1998; García-Navas and Sanz, 2010; Mathot et al., 2017), provisioning frequency and food allocation (Christe et al., 1996; Naef-Daenzer and Keller, 1999; Wilkin et al., 2009; Mutzel et al., 2013; van Oers et al., 2015; Caro et al., 2016). Early environmental conditions have been extensively studied in altricial birds, since offspring may experience variable natural environmental conditions that are easily manipulated experimentally, such as brood size. The effects of brood size are likely due to nutritional stress, but the direct causes of nutritional stress may depend on the provisioning tactics of the parents (Mathot et al., 2017). Parents may be unable to compensate for an increased food demand (Gow and Wiebe, 2014; Mathot et al., 2017) or may increase the feeding frequency (Hinde and Kilner, 2007; Baldan et al., 2019) but reduce prey selectivity in enlarged broods (Wright et al., 1998; García-Navas and Sanz, 2010; Mathot et al., 2017). Another cause of nutritional stress could be an increase in nestling begging costs in enlarged broods (Neuenschwander et al., 2003) due to increased social stress and competition. Brood size most prominently modifies offspring growth (Tinbergen and Boerlijst, 1990; Naguib et al., 2004; Nettle et al., 2013) and development (Naguib et al., 2004), in turn affecting fledging age (Naguib et al., 2004) and fledging size/condition (Tinbergen and Boerlijst, 1990; Sanz and Tinbergen, 1999). However, brood size has also an effect on offspring physiology, where larger brood sizes cause changes in energy metabolism (Mertens, 1969), immunocompetence (Brinkhof et al., 1999; Saino et al., 2003; Naguib et al., 2004), testosterone levels (Naguib et al., 2004), the stress response (Naguib et al., 2011) and corticosterone levels (Saino et al., 2003). Ultimately, these changes have consequences for the cognitive ability (Nettle et al., 2015) and the behavior (Carere et al., 2005; Krause et al., 2009) of offspring. However, not much is known about how these early developmental conditions shape development, physiology and behavior of the offspring in a stable manner. Changes in DNA methylation due to these early developmental conditions are a good candidate for explaining parental induced phenotypic

plasticity, but only few studies examined early environmental effects on DNA methylation in wild avian populations (Bentz et al., 2016; Rubenstein et al., 2016; Sheldon et al., 2018; Jimeno et al., 2019; Sepers et al., 2019). Only two of these studies made use of a brood size experiment. In a study on captive zebra finches (*Taeniopygia guttata*) individuals reared in large broods showed higher DNA methylation of the glucocorticoid receptor gene (*Nr3c1*) compared to individuals raised in small broods (Jimeno et al., 2019). Since only one candidate gene was targeted, it remains to be elucidated if more genes are affected. In another study on the same species, however, no difference in DNA methylation was detected when comparing experimentally reduced and enlarged broods using MS-AFLP (Sheldon et al., 2018). The chosen method (MS-AFLP; Reyna-López et al., 1997) has some drawbacks, it for example only screens anonymous loci and since no annotation is possible, no clear expectations can be formulated (Schrey et al., 2013). In the study of Sheldon et al. (2018), only the enlarged and reduced broods were manipulated, leaving the control broods unmanipulated. Manipulated individuals showed more hypomethylation compared to unmanipulated individuals, suggesting an effect of manipulation on DNA methylation (Sheldon et al., 2018). Thus, early developmental conditions might induce changes in nestling DNA methylation via post-natal effects such as brood size, however, to what extent early life conditions causally affect DNA methylation in functionally relevant genes is largely unknown.

Here, we experimentally manipulated brood size and assessed its effect on DNA methylation in a wild songbird species, the great tit (*Parus major*). The great tit has been a model system for ecological and evolutionary studies, with long-term studies in both wild and captive populations (Laine et al., 2016; Bosse et al., 2017; Spurgin et al., 2019). We cross-fostered 3-day old nestlings between pairs of matched broods creating enlarged broods with three nestlings extra and reduced broods with three nestlings less. In the two broods within a control pair, the original brood size of both broods remained the same, but half of the nest was cross-fostered. This classical approach has been shown to be an effective way to affect offspring behavior, physiology and body size (Sanz and Tinbergen, 1999; Neuenschwander et al., 2003; van Oers et al., 2015) and allows us to disentangle prehatching from rearing effects (Sepers et al., 2019). In birds, like most vertebrates, almost all methylation occurs at CpG sites (Derks et al., 2016; Laine et al., 2016). DNA methylation variation in the great tit has been associated with phenotypic traits such as exploratory behavior (Riyahi et al., 2015; Verhulst et al., 2016) and the onset of reproduction (Viitaniemi et al., 2019; Lindner et al., 2021a). Low levels of CpG promoter region methylation, and more specifically of sites in the transcription start site (TSS), are associated with increased gene expression in the great tit (Laine et al., 2016). We therefore used Reduced Representation Bisulfite Sequencing (RRBS) to compare CpG site-specific DNA methylation levels between siblings from enlarged and reduced broods, and between siblings from control broods. Furthermore, we used the existing annotation of the great tit reference genome to assess the functional importance of differentially methylated sites.

MATERIALS AND METHODS

Subjects, Study Site, and General Procedures

This study was conducted in April, May and June 2016 in the Boslust study population, near Arnhem, Netherlands (5°8'50" E, 52°0'10" N), a 70 ha field site consisting of mixed pine-deciduous woodlands and grassy meadows. The study site contained about 150 nest boxes, which were predominantly used by great tits. From the first week of April onward, we checked nest boxes weekly to determine initiation of breeding activity. We inspected occupied nest boxes every other day to determine the date of first egg-laying, clutch size and start of incubation, allowing us to estimate hatch dates. By visiting nests daily around the expected hatch date, we determined the exact date at which the majority of the eggs within a clutch hatched (hereafter: hatch date).

Cross-Fostering and Brood Size Manipulation

Clutches with the same hatching date (D0) and similar brood sizes were assigned to a cross-foster pair ($N = 30$ broods; 15 cross-foster pairs). A cross-foster pair was randomly assigned to become either a control pair or a treatment pair, independently of original brood size. When nestlings were three days old (D3), a partial cross-foster design was employed. We used the method according to van Oers et al. (2015) for cross-fostering. For this, nestlings within broods were ranked based on their weight (using a digital scale, ± 0.01 g) and then randomly either the even or the odd ranked nestlings were swapped between the two broods (Supplementary Figure 1). In this way, differences in weight between cross-fostered nestlings and nestlings that stayed in the brood of origin were minimized (van Oers et al., 2015). For control pairs (twelve control broods; six cross-foster pairs), half of the nestlings were swapped (cross-fostered) between the two broods, while the other half stayed in the brood of origin (unmoved), without changing the original brood size. For treatment pairs (nine reduced and nine enlarged broods; nine cross-foster pairs), one brood received three nestlings more than the original brood size (+3, enlarged) and the other brood received three nestlings less than the original brood size (−3, reduced) (Supplementary Figure 1). We aimed for similar numbers of unmoved compared to cross-fostered nestlings in a brood and minimal weight differences between unmoved and cross-fostered siblings.

To be able to identify individuals and their brood of origin, the down tufts on the head, wings and back of the nestlings were selectively plucked right before weighing and cross-fostering (van Oers et al., 2015). This enabled us to identify the nestlings up until day six, the day at which nestlings were fitted with uniquely numbered aluminum bands (Vogeltrekstation, Netherlands).

Fourteen days after hatching (D14), a blood sample (approximately 10 μ L) was taken from the brachial vein and stored in Eppendorf tubes containing one ml cell lysis buffer. The tubes were stored at the NIOO-KNAW at room temperature until further analysis. Since some broods were deserted or nestlings were missing or found dead, we were able to take blood

samples from 153 nestlings from 25 broods. Of these 25 broods nine were control broods (three complete control pairs and three single control broods) and seven were enlarged broods and nine were reduced broods (seven complete treatment pairs and two single treatment broods).

Sample Selection and Processing

From the seven treatment pairs (reduced and enlarged), matched samples from two biological siblings ($N = 14$; seven reduced and seven enlarged) were chosen for further analysis. To disentangle treatment effects on DNA methylation from biological variation in DNA methylation between the enlarged and reduced pool, we decided to compare two control pools as well. From the three control pairs, four samples ($N = 12$) per cross-foster pair were chosen for further analysis (biological sibling pairs being raised in different control broods). Since siblings are more similar to each other in their methylation profile than to nestlings from other broods (Viitaniemi et al., 2019; van Oers et al., 2020), this approach allowed us to control for prehatching differences in DNA methylation by only comparing DNA methylation levels between siblings raised in enlarged and reduced broods and between siblings raised in control broods. This approach resulted in a total sample size of 26 samples from 26 individuals (Supplementary Table 1).

Reduced Representation Bisulfite Sequencing Library Preparation and Sequencing

For DNA isolation, red blood cells were separated from the plasma by spinning the samples in a centrifuge at 14,000 rpm for twelve minutes. Subsequently, the plasma was removed using Hamilton syringes (Merck KGaA) and the DNA was extracted from the red blood cells using FavorPrepT M 95-well Genomic DNA Kit. After DNA extraction, the DNA concentration of each individual sample was checked on a NanoDrop 2000 spectrophotometer (Thermo Scientific, Waltham, MA, United States). If needed, a sample was diluted to equalize the concentrations of all the samples. In addition, DNA quality was checked on agarose gels. Subsequently, the samples were pooled per treatment, resulting in a Reduced pool and an Enlarged pool (both consisting of seven individuals), and a Control1 pool and a Control2 pool (both consisting of six individuals) (Supplementary Table 1). The samples were pooled based on concentration, in order to minimize variation in the amount of starting DNA between individuals in one pool. Earlier research showed that pooling individuals is a reliable way to assess average group DNA methylation (Docherty et al., 2009). Within one pool all samples were, to our knowledge, unrelated. DNA isolation was finalized within one day to prevent batch effects between pools. We assessed genome-wide DNA methylation levels using Reduced Representation Bisulfite Sequencing (RRBS; Meissner, 2005). RRBS was done as mentioned in Derks et al. (2016). The genomic DNA was digested with the enzyme Msp1. This enzyme cuts the genomic DNA at CCGG motif sites. The restriction fragments were size selected to a range of 20–200 bp, by cutting from gel after preparative gel electrophoresis.

Secondly, the fragmented DNA was treated with the chemical sodium bisulfite, which turns unmethylated cytosines (C's) into uracils (U's) which will later be read as thymines (T's). Bisulfite-PCR amplification was conducted using PfuTurboCx Hotstart DNA polymerase (Stratagene) and 18 PCR cycles. The final amplified Enlarged and Reduced pools were sequenced in 2016 on an Illumina HiSeq 2500 (100 bp from single end reads), and the final amplified control pools were sequenced in 2018 on an Illumina HiSeq 4000 (100 bp from single end reads). Bisulfite sequencing was done by the Roy J. Carver Biotechnology Centre (University of Illinois at Urbana-Champaign, United States).

DNA Methylation Analysis

Quality Control and Trimming

Raw reads from the RRBS data were quality checked and checked for adapter content using FastQC v0.11.8 (Andrews, 2010). FastQ screen v0.11.1 (Wingett and Andrews, 2018) in bisulfite mode was used to detect possible contaminations with pre-existing databases and indexed genomes. The databases and genomes were Phix (*Coliphage phi-X174*), vectors (UniVec Core), *Arabidopsis thaliana* (*A. thaliana* (thale cress), TAIR10), *Escherichia coli* (*E. coli str. K-12 substr. MG1655*) and *Homo sapiens* (Genome Reference Consortium Human Build 38). After assessment of the quality checks, measures were taken to improve the quality of the reads. For this, the reads were trimmed for quality (≥ 20 PHRED quality score), length (≥ 20 bp for the control pools and ≥ 36 bp for the treatment pools because of high per base N content) and adapter sequences using Trim Galore v0.4.1 (Krueger, 2012) with the `--rrbs` option. The results were summarized using Multiqc 1.7 (Ewels et al., 2016). Quality improvement of the reads was verified by FastQC, FastQ screen and Multiqc. Raw reads were submitted to NCBI under the BioProject PRJNA208335 with accession numbers SRR11078101 (Enlarged pool), SRR11078100 (Reduced pool), SRR11078099 (Control1) and SRR11078098 (Control2).

Alignment and Methylation Calling

Trimmed reads were aligned to the *Parus major* reference genome v1.1¹ (Laine et al., 2016) using BS-Seeker2² v2.0.6 (Guo et al., 2013) with Bowtie2³ v2.1.0 (Langmead et al., 2009) using the end-to-end alignment mode. Of the aligned reads, the methylation levels for each site were determined by dividing methylated C's of a site by the total coverage of that site (C/C + T) which was done with the methylation call script `bs_seeker2-call_methylation.py` from BS-Seeker2.

Filtering of Methylation Calls

Before the data was filtered it was transformed to fit the format of methylKit. This was done using a custom bash script and only for CpG sites. Filtering was done with R version 3.6.1 and the R package methylKit⁴ v1.15.3 (Akalin et al., 2012). First, a principle component analysis (PCA) was conducted on all CpG

¹https://www.ncbi.nlm.nih.gov/assembly/GCF_001522545.3

²<https://github.com/BSSeeker/BSseeker2>

³<http://bowtie-bio.sourceforge.net/bowtie2>

⁴<https://github.com/al2na/methylKit>

sites that were present in all pools to check clustering of the four pools. The default settings of the *PCASamples* function in methylKit were used, which means that the percent methylation matrix was transposed (this is equivalent to doing PCA on variables that are sites) and that sites with low variation in DNA methylation or low coverage (<10) were discarded prior to the PCA. To test whether average CpG methylation percentage differed significantly between the four pools, a one-way analysis of variance (ANOVA) and subsequent Tukey *post hoc* analyses were conducted. Next, sites that were not present in both pools (both treatment pools or both control pools), sites with low coverage (<10) and sites with methylation levels of 0 or 100% in all pools were excluded. The treatment pools were sequenced deeper (i.e., higher coverage) than the control pools and to avoid a PCR bias in the statistical tests, we applied percentile filtering (99.9) and the coverage of the samples was normalized. After filtering of the control pools, 213,764 out of the 247,979 CpG sites that were present in both control pools could be used for further analysis. After filtering of the treatment pools, 235,618 out of the 252,698 CpG sites that were present in both treatment pools could be used for further analysis. Correlation matrixes were made to check for abnormalities.

Statistical Analysis

Differentially methylated sites (DMS) between Reduced and Enlarged and between Control1 and Control2 were also assessed using the R package methylKit v1.15.3 and R version 3.6.1 (Akalin et al., 2012). MethylKit reads the data and creates a data frame where it calculates the percentage of methylated C's at a given site from the methylation ratio created by BSSeeker2. Complementary CpG dinucleotides were not merged. Next, differential methylation per site was assessed by comparing the fraction of methylated C's between two pools using a Fisher's exact test, since there was only one pool per group for both comparisons. To minimize the chance of getting false positives, we decided to use a stringent threshold of 25% instead of 10% differential methylation. We used a Bonferroni corrected α -threshold [$-\log_{10}(0.05/213,764) = 6.63$ for Control1 vs. Control2 and $-\log_{10}(0.05/235,618) = 6.67$ for Reduced vs. Enlarged] for a site to be considered a DMS.

Gene Annotation

DMS were annotated using the *Parus major* reference genome build v1.1⁵, annotation version 102⁶, custom R scripts and R packages GenomicFeatures v1.30.0 (Lawrence et al., 2013) and rtracklayer v1.42.2 (Lawrence et al., 2009). Genomic regions were TSS, promoter, intron, exon, five prime untranslated region (5'UTR), three prime untranslated region (3'UTR), upstream and downstream. TSS regions were defined as 300 bp upstream to 50 bp downstream of the annotated transcription starting position of each gene (Laine et al., 2016; Viitaniemi et al., 2019). Since TSS regions overlap with promoter regions, DMS associated to a TSS region were also associated to a promoter region. In such cases, only the TSS region was reported. We

defined the TSS region as in Laine et al., 2016 since in this study, low levels of CpG methylation in specifically this region were associated with increased gene expression in the great tit. Promoter regions were defined as 2000 bp upstream to 200 bp downstream of the annotated gene start site (Lindner et al., 2021a). Upstream and downstream regions were defined as 10 K bp up- and downstream regions adjacent to the gene body, respectively (Laine et al., 2016; Lindner et al., 2021b). Since DNA methylation is reciprocal on both strands, annotation was not directional (i.e., each DMS could overlap with the Watson and Crick strands). Please note that one site can be associated to multiple genes or regions, because genes can be located on opposite strands, regions within a gene can have overlapping regions depending on the transcript and genes can have opposite transcription directions. If this was the case, we checked the site in NCBI Genome Data Viewer and prioritized DMS in TSS and promoter regions, because CpG methylation within the regulatory region is known to affect gene expression in great tits (Laine et al., 2016).

Gene Ontology Analysis

The function and significance of the genes that were associated to a DMS were investigated by looking up GO terms and descriptions of the genes of chicken at uniprot⁷, NCBI⁸, Ensembl⁹ and genecards¹⁰. We focused on molecular functions and biological processes. If there were no GO terms and descriptions of chicken (*Gallus gallus*) genes available, we used zebra finch (*T. guttata*) or human genes (*Homo sapiens*). Uncharacterized LOC genes were checked using NCBI and Ensembl. A LOC gene was included if its biological function could be predicted and was excluded if it was likely to be a duplication of (part of) the gene it was predicted to be, ncRNA or truly uncharacterized. We focused on DMS within regulatory regions (promoter and TSS regions) of genes and DMS that occurred in the same gene. Information about other DMS can be found in **Supplementary Tables 4–9**.

Additionally, GOrilla was used to identify enriched GO terms (Eden et al., 2009). Since there was not enough power to provide both a background and a target list, all genes in which a CpG site was found, so all genes that were covered by both pools in one comparison, were given as input. This was done for the Enlarged pool versus the Reduced pool comparison and the Control1 pool versus Control2 pool comparison separately. The genes were ranked according to how well the associated CpG site differentiated between the two pools that were compared using the *p*-value from the Fisher's exact test as described above. LOC genes were excluded by GOrilla. GOrilla was run with default running parameters (species used: *H. sapiens*; single ranked list of genes, *p*-value <0.001 , GO database last updated on December 12, 2020). The FDR method was used to correct enrichment tests for multiple testing of the GO terms. GO categories were considered significantly enriched if the FDR corrected *p*-value was <0.05 .

⁷www.uniprot.org

⁸www.ncbi.nlm.nih.gov

⁹www.ensembl.org

¹⁰<http://www.genecards.org>

⁵https://www.ncbi.nlm.nih.gov/assembly/GCF_001522545.3

⁶https://ftp.ncbi.nlm.nih.gov/genomes/all/annotation_releases/9157/102/

All scripts used for the bioinformatics and biostatistics steps can be found in the **Supplementary Material**.

RESULTS

General

The percentage of fully bisulfite converted reads was >99.99% in all pools (**Supplementary Table 2**). A Principle Component Analysis (PCA) revealed that 51.95% was explained by PC1, 39.07% by PC2, 8.98% by PC3 and $2.20 \times 10^{-29}\%$ by PC4 (**Supplementary Figure 2**). The Reduced pool and the Enlarged pool cluster together very closely along both PC1 and PC2 (**Supplementary Figure 3**). The two control pools are relatively close to one another along PC2 but vary along PC1.

Average CpG methylation percentage differed significantly between the four pools (one-way ANOVA: $F_{3,836192} = 96.8$, $p < 0.02 \times 10^{-14}$). A Tukey *post hoc* test showed that all pools differed significantly in average CpG methylation percentage: the methylation percentage was significantly lower in the Reduced pool compared to the Enlarged pool (mean \pm SE; Reduced:

27.48 ± 0.0007 ; Enlarged: 27.76 ± 0.0007 ; $p = 0.04$). The methylation percentage was significantly higher in Control1 compared to Control2 (Control1: 29.13 ± 0.0008 ; Control2: 28.41 ± 0.0007 ; $p = 0.07 \times 10^{-9}$). The methylation percentages in the treatment pools were significantly lower compared to the control pools (all $p \leq 0.04 \times 10^{-7}$).

An overview of the number of reads and CpG sites before and after filtering and the mapping and calling success is given in **Supplementary Table 3**. 235,618 CpG sites were present in both treatment pools and 213,764 CpG sites were present in both control pools (after filtering). 209,049 CpG sites were shared by all four pools (**Supplementary Figure 4**).

Of the 213,764 CpG sites that were present in both control pools, 17 sites were significantly differentially methylated (**Figure 1A**), of which 14 were located on autosomes, two on the sex chromosome (chrZ) and one on a scaffold (**Figure 2A**). Of the 17 DMS, 14 sites could be annotated. Of these 14 DMS, four were located in a promoter region, of which two were located in a TSS region. Furthermore, one DMS was located in an exonic region, five in an intronic region, two in a downstream region, one in an upstream region and one DMS in both an upstream

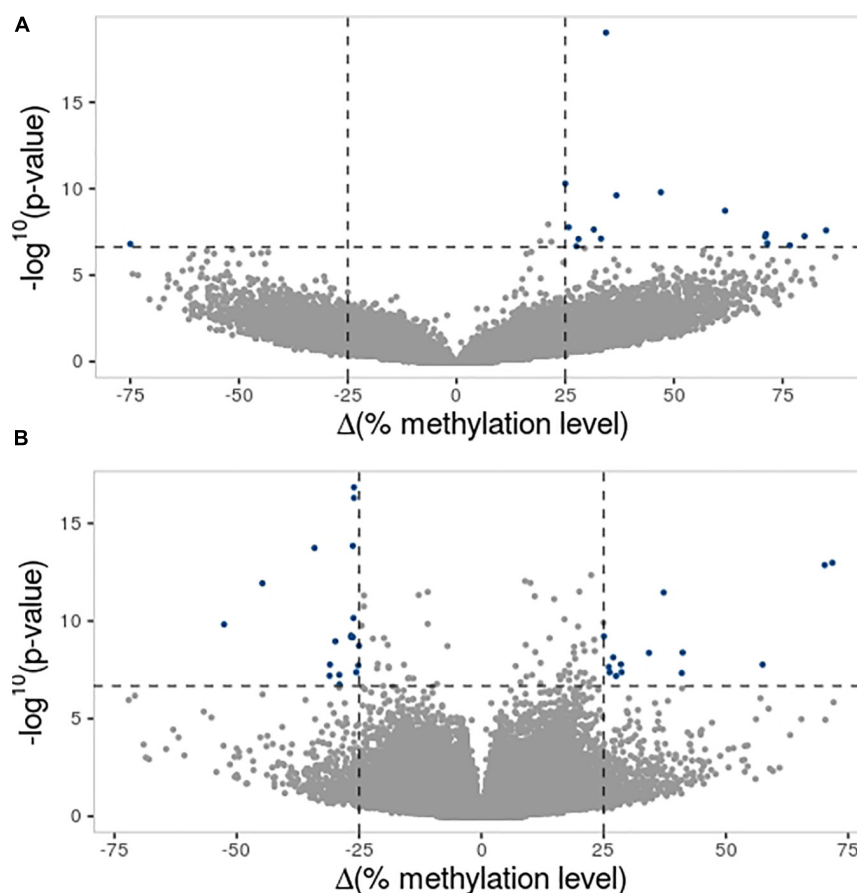
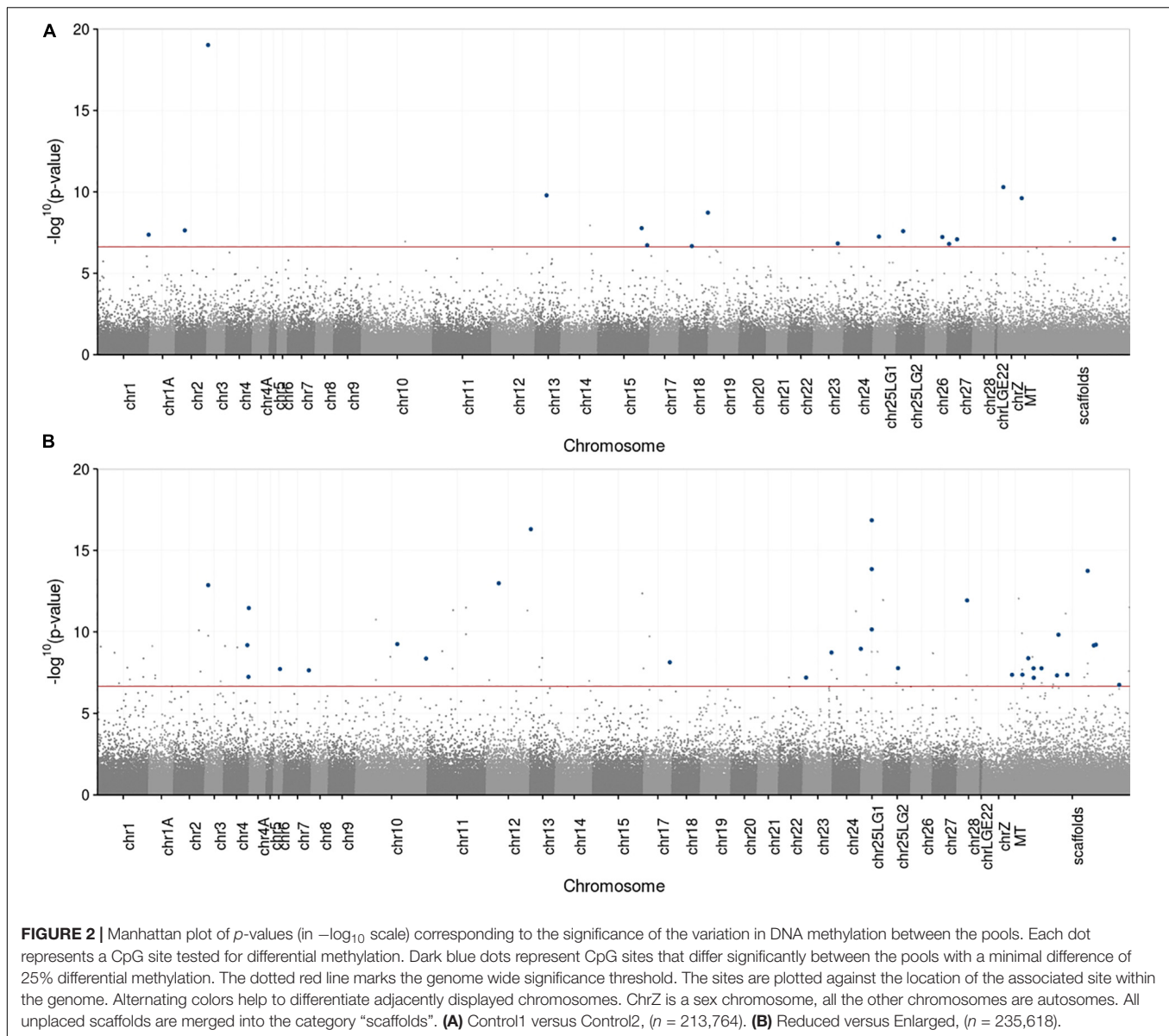


FIGURE 1 | Volcano plot of p -values (in $-\log_{10}$ scale) corresponding to the significance of the variation in DNA methylation between two pools. Each dot represents a CpG site tested for differential methylation. Dark blue dots represent CpG sites that differ significantly between the pools with a minimal difference of 25% differential methylation. Dotted horizontal lines marks the genome wide significance threshold. Dotted vertical lines represent 25% differential methylation. **(A)** Control1 versus Control2, ($n = 213,764$). **(B)** Reduced versus Enlarged, ($n = 235,618$).



and downstream region (**Supplementary Table 4**). The 14 DMS were located in or nearby 15 genes (**Supplementary Table 5**).

Of the 235,618 CpG sites that were present in both treatment pools, 32 sites were significantly differentially methylated (**Figure 1B**), of which 20 were located on autosomes, one on the sex chromosome and 12 on scaffolds (**Figure 2B**). Of the 32 DMS, 23 could be annotated. Of these 23 DMS, nine were located in a promoter region, of which three in a TSS region. Furthermore, three DMS were located in an exonic regions, eight DMS were located in an intronic region, two in a downstream region and one in an upstream region (**Supplementary Table 4**). The 23 DMS were located in or nearby 21 genes (**Supplementary Table 6**).

None of the 17 DMS between Control1 pool and Control2 pool were significantly differentially methylated between the Reduced pool and the Enlarged pool (**Supplementary Figure 5**

and **Supplementary Table 7**). Out of the 17 DMS, 11 were covered in the Reduced pool and the Enlarged pool. None of the 32 DMS between the Reduced pool and the Enlarged pool were significantly differentially methylated between Control1 pool and Control2 pool (**Supplementary Figure 5** and **Supplementary Table 7**). Out of the 32 DMS, 26 were covered in both Control pools.

Differentially Methylated Sites in Genes and in Regulatory Regions Control Pools

When comparing the two control pools, none of the DMS occurred within the same genes. All of these DMS were hypermethylated, which translates to a higher methylation percentage in Control1 compared to Control2. We found four

TABLE 1 | Full name, gene name, location, chromosome number or unplaced scaffold, Δ of % methylation level, p -value, region and biological functions associated with DMS between Control1 pool and Control2 pool.

Location DMS (chrom./scaf.)	Δ meth. (p -value)	Region	Biological functions
Heat shock protein family A (Hsp70) member 2 (<i>HSPA2</i>)			
54944183 (chr5)	26% (1.68×10^{-8})	Promoter	Protein refolding, spermatogenesis
Methyltransferase like 8 (<i>METTL8</i>)			
17727814 (chr7)	28% (2.12×10^{-7})	Promoter	Skeletal muscle tissue development, fat cell differentiation, histone acetylation, mRNA methylation
Zinc finger SWIM domain-containing protein 1 (<i>ZSWIM1</i>-like; <i>LOC107213098</i>)			
6759952 (chr20)	28% (8.15×10^{-8})	TSS	Zinc and metal ion binding
Oncostatin-M-specific receptor subunit beta-like (<i>OSMR</i>-like; <i>LOC107198170</i>)			
11977179 (chrZ)	25% (5.05×10^{-11})	TSS	Cell population proliferation, inflammatory response

DMS to be situated in regulatory regions (Table 1). These DMS were located in the promoter region of heat shock protein family A member 2 (*HSPA2*), in the promoter region of methyltransferase like 8 (*METTL8*), around the TSS region of the predicted gene zinc finger SWIM domain-containing protein 1 (*ZSWIM1*-like; *LOC107213098*) and around the TSS region of the predicted gene oncostatin-M-specific receptor subunit beta-like (*OSMR*-like; *LOC107198170*).

Out of all 15,603 covered genes, 11,182 were recognized by GOrilla. We detected 98 enriched GO terms for the ontology biological process, 28 for the molecular functions and 16 for cellular component (p -value <0.001). After FDR correction, 47 of the biological function GO terms were significantly enriched, 19 of the molecular function and eight of the cellular component (FDR q -value <0.05) (Supplementary Table 10). The most significant GO terms were anatomical structure development (GO:0048856, FDR = 1.95×10^{-8}), developmental process (GO:0032502, FDR = 7.28×10^{-6}) and system development (GO:0048731, FDR = 5.09×10^{-5}). Of all genes (excluding the LOC-genes) in which a DMS was found, only *HSPA2* was associated with the significantly enriched GO terms.

Reduced Versus Enlarged

In the treatment comparison, three DMS were found within the same gene (Table 2). These three DMS were hypomethylated, which translated to a lower methylation percentage in the Enlarged pool than in the Reduced pool. Of these three DMS, one was situated in the exonic region and two in the intronic region of laminin subunit gamma 3 (*LAMC3*). Nine DMS were situated in the regulatory regions of genes and none of these DMS occurred in the same gene (Table 2). Of these nine DMS, five were hypermethylated, which translated to a higher methylation percentage in the Enlarged pool than in the Reduced pool. These DMS were situated around the TSS region of tissue specific transplantation antigen P35B (*TSTA3*), around the TSS region of the predicted gene ketosamine-3-kinase-like (*FN3KRP*-like; *LOC107198385*), in the promoter region of prominin 2 (*PROM2*), around the TSS region of zinc finger protein 664-like (*ZNF664*-like; *LOC107199222*) and in the promoter region of plectin-like (*PLEC*-like; *LOC107199333*). The four remaining DMS in regulatory regions of genes were hypomethylated, which translated to a lower methylation percentage in the Enlarged pool than in the Reduced pool. These DMS were situated in

the promoter region of complement C1q subcomponent subunit C-like (*C1QC*-like; *LOC107213704*), in the promoter region of the gene activating transcription factor 6 (*ATF6*), in the promoter region of prolactin regulatory element binding (*PREB*) and in the promoter region of WD repeat domain 83 opposite strand (*WDR83OS*).

Out of all 15,718 covered genes, 11,210 were recognized by GOrilla. We detected 82 enriched GO terms for the ontology biological process, 23 for the molecular functions and seven for cellular component (p -value <0.001). After FDR correction, 42 of the biological function GO terms were significantly enriched, eleven of the molecular function and one of the cellular component (FDR q -value <0.05) (Supplementary Table 11). The most significant GO terms were developmental process (GO:0032502, FDR = 1.64×10^{-5}), regulation of multicellular organismal process (GO:0051239, FDR = 6.72×10^{-5}), anatomical structure morphogenesis (GO:0009653, FDR = 4.71×10^{-5}), anatomical structure development (GO:0048856, FDR = 1.09×10^{-4}) and regulation of cell differentiation (GO:0045595, FDR = 9.59×10^{-5}). All genes (excluding the LOC-genes) in which a DMS was found were associated with the significantly enriched GO terms.

The results that were obtained with a less stringent threshold of 10% differential methylation are reported in Supplementary Tables 12–16.

DISCUSSION

The mechanisms underlying intergenerational regulation of developmental phenotypic plasticity in birds remain largely unknown, but recent studies indicate a role for DNA methylation (Bentz et al., 2016; Sheldon et al., 2018). Here, we explored this further by experimentally manipulating brood size in a partial cross-foster experiment and assessing the effect of experimental brood size on DNA methylation in a wild songbird species, the great tit. We found more CpG sites in red blood cells to be differentially methylated between biological sibling-pairs raised in experimentally enlarged and reduced broods, than between siblings raised in partially cross-fostered control broods with unchanged brood size. Since differential DNA methylation is more apparent between nestlings from enlarged and reduced broods than between nestlings from control broods,

TABLE 2 | Full name, gene name, location, chromosome number or unplaced scaffold, Δ of % methylation level, p -value, region and biological functions associated with DMS between the Reduced pool and the Enlarged pool.

Location DMS (chrom./scaf.)	Δ meth. (p-value)	Region	Biological functions
Laminin subunit gamma 3 (LAMC3)			
4729995 (chr17)	−26 (1.42 × 10 ^{−14})	Intron	Cell morphogenesis, cell differentiation, visual perception, astrocyte development, retina development in camera-type eye
4730000 (chr17)	−26 (7.06 × 10 ^{−11})	Intron	
4730148 (chr17)	−26 (1.42 × 10 ^{−17})	Exon	
Tissue specific transplantation antigen P35B (TSTA3)			
149822731 (chr2)	34 (4.28 × 10 ^{−9})	TSS	T cell mediated cytotoxicity, nucleotide-sugar biosynthetic process
Complement C1q subcomponent subunit C-like (C1QC-like; LOC107213704)			
1797839 (chr21)	−45 (1.17 × 10 ^{−12})	Promoter	Negative regulation granulocyte differentiation, negative regulation macrophage differentiation, synapse pruning
Prominin 2 (PROM2)			
730012 (chr22)	70 (1.36 × 10 ^{−13})	Promoter	Cell projection organization, protein phosphorylation, regulation of GTPase activity (signal transduction)
Ketosamine-3-kinase-like (FN3KRP-like; LOC107198385)			
70917608 (chrZ)	26 (4.27 × 10 ^{−8})	TSS	Post-translational protein modification (phosphorylation)
Activating transcription factor 6 (ATF6)			
246819 (scaffold)	−29 (1.77 × 10 ^{−7})	Promoter	Eye development, regulation transcription, unfolded protein response (endoplasmic reticulum stress), cell apoptosis
Prolactin regulatory element binding (PREB)			
50860 (scaffold)	−34 (1.82 × 10 ^{−14})	Promoter	Protein exit from endoplasmic reticulum
Zinc finger protein 664-like (ZNF664-like; LOC107199222)			
19200 (scaffold)	29 (4.18 × 10 ^{−8})	TSS	–
WD repeat domain 83 opposite strand (WDR83OS)			
11884 (scaffold)	−53 (1.49 × 10 ^{−10})	Promoter	Phosphorylation, MAPK cascade, (m)RNA splicing, mRNA processing
Plectin-like (PLEC-like; LOC107199333)			
1772 (scaffold)	28 (6.52 × 10 ^{−8})	Promoter	–

–, The biological function could not be predicted.

this indicates that experimental variation in brood size affects DNA methylation. Furthermore, we found for the enlarged versus reduced comparison more differentially methylated CpG sites to be situated in regulatory regions (promoter and TSS regions) than for the control brood comparison. Since CpG methylation within the regulatory region is known to affect gene expression in great tits (Laine et al., 2016), we expect more functional differences in gene expression between nestlings from enlarged and reduced broods than between nestlings from control broods.

The average CpG methylation percentage differed between pools of nestlings from the enlarged and the reduced broods and between the control broods. Nestlings from experimentally enlarged and reduced broods were hypomethylated compared to nestlings from control broods. This indicates that any manipulation of brood size affected CpG methylation. A similar result was found in the study of Sheldon et al. (2018). Here, zebra finch nestlings from reduced and enlarged broods showed more hypomethylation compared to control nestlings. However, in this study, the control broods were completely unmanipulated, whereas in our study nestlings from control broods were also cross-fostered. Moreover, we found that nestlings from experimentally enlarged broods were hypermethylated compared to nestlings from reduced broods. This result supports the hypermethylation of Nr3c1 in zebra finches reared in large broods (Jimeno et al., 2019) and the positive correlation between natal

brood size and the percentage of DNA methylation in Sheldon et al. (2018). However, this result does not match the lack of difference in methylation between nestlings from experimentally reduced and enlarged broods (Sheldon et al., 2018). This might be caused by the targeted approach we used in this study, compared to the MS-AFLP approach. In spite of our targeted approach, slight differences in methylation remain undetected when average DNA methylation levels are compared. Since DNA methylation is very gene- and region specific, it is important to assess site specific differences in possibly functionally relevant genes as well. Furthermore, the difference in methylation percentage between nestlings from reduced and enlarged broods was only 0.28% and the difference between nestlings that experienced a manipulation in brood size and nestlings from control broods ranged from 0.65 to 1.65%, also minimal differences. It has to be elucidated if such small differences are large enough to result in differential gene expression. Hence, it is unknown if such small differences hold any biological significance or are just caused by a statistical artifact.

As mentioned above, the biological functions of the genes and the possible consequences of methylation for gene expression will be discussed below. Since low levels of CpG promoter region methylation, and more specifically of sites in the TSS region, are associated with increased gene expression in the great tit (Laine et al., 2016), hypermethylated DMS are expected to be

associated with lower gene expression and hypomethylated DMS are expected to be associated with higher gene expression. The limitations of such a generalization will also be discussed.

Sites that were differentially methylated in the enlarged versus reduced comparison, were mainly found to be related to development, metabolism and behavior and cognition. This involved sites in the genes *LAMC3*, *PREB*, *PROM2*, *TSTA3*, *ATF6*, *FN3KRP*-like and *WDR83OS*. The biological functions of *ZNF664*-like and *PLEC*-like could not be predicted because these LOC genes were likely to be a duplication of (part of) the genes they were predicted to be or ncRNA. In the gene *LAMC3*, three different DMS were found, although none of them occurred in the regulatory region of the gene, indicating that we have no proof for a possible change in gene expression in the great tit (Laine et al., 2016). The DNA methylation levels in all three sites were higher in the reduced pool in comparison with the enlarged pool, cautiously indicating lower expression of *LAMC3* in nestlings from reduced broods. Since *LAMC3* expression is relatively high during human development (Barak et al., 2011) and low to moderate in adulthood (Hawrylycz et al., 2012; Zeng et al., 2012), this might mean that the nestlings from the reduced broods were further developed than the nestlings from the enlarged broods, which is in the expected direction based on previously found effects of brood size and food availability on development (Nettle et al., 2013) and condition (Tinbergen and Boerlijst, 1990; DeKogel, 1997; Sanz and Tinbergen, 1999). In addition, we found one DMS in the genes *PREB*, *PROM2*, *TSTA3*, *ATF6*, *FN3KRP*-like and *WDR83OS*. The DMS in both *PROM2* and *TSTA3* were hypermethylated in the enlarged pool compared to the reduced pool, suggesting lower gene expression in nestling from the enlarged broods. In the case of *PROM2*, this might indicate lower levels of cholesterol (Singh et al., 2013) in the nestlings from enlarged broods. In the case of *TSTA3*, this might indicate a lower growth potential (Willson et al., 2018) of nestlings in the enlarged broods and an effect on (bone) metabolism (Johnsson et al., 2015). The DMS in *PREB* was hypomethylated in the enlarged group, suggesting higher expression of these genes in nestlings from the enlarged group. Higher *PREB* expression might lead to lower prolactin (PRL) expression (Hiyama et al., 2015), which might indicate later sexual maturity and lower body weights (Bhattacharya et al., 2011) in nestlings from the enlarged broods. Overall, the DMS in *PREB*, *PROM2* and *TSTA3* indicate that the nestlings from the enlarged broods weighed less, developed slower and adjusted their metabolism. These effects are in the expected direction based on previously found effects of brood size on nestling condition (DeKogel, 1997; Sanz and Tinbergen, 1999), weight (Tinbergen and Boerlijst, 1990; DeKogel, 1997) and resting metabolic rate (Verhulst et al., 2006).

The genes *ATF6*, *PREB*, *FN3KRP*-like and *WDR83OS* suggest an effect of brood size on insulin-glucose metabolism specifically. The DMS in *PREB*, *ATF6* and *WDR83OS* were hypomethylated in the enlarged pool compared to the reduced pool, suggesting higher gene expression in nestlings from the enlarged broods. This indicates in the case of *ATF6* glucose intolerance (Barbosa et al., 2016), in the case of *WDR83OS* increased levels of insulin (Kesharwani et al., 2017) and in the case of *PREB* higher insulin sensitivity (Park et al., 2018) in nestlings from

the enlarged broods. The function of *FN3KRP*-like function is not fully understood (Szwergold et al., 2011), although the hypermethylated DMS in the enlarged pool suggests lower gene expression in nestlings from the enlarged broods, which indicates differences in glucose metabolism (Sajuthi et al., 2016) between nestlings from the reduced and enlarged broods. The effects are in the expected direction based on previously found effects of brood size on energy metabolism (Mertens, 1969) and the results indicate food scarcity in the enlarged broods. The specific effect of food scarcity on insulin-glucose metabolism has to be elucidated yet, because the effect is dependent on the developmental stage of an individual (Gardner et al., 2005; Tobi et al., 2009). Nevertheless, insulin-glucose metabolism might be a way of dealing with nutritional constraint in the enlarged broods, allowing for growth under poor food conditions (Gardner et al., 2004, 2005). Similar results have been found before. For example, women exposed to the Dutch famine during gestation gave birth to individuals with lower glucose tolerance during adulthood, probably caused by impaired insulin secretion (de Rooij et al., 2006). This is thought to be the result of fetal adaptations to scarcity i.e., the thrifty phenotype (Hales and Barker, 1992), which becomes maladaptive when an individual is exposed to an abundance of food later in life (Stanner and Yudkin, 2001; Schulz, 2010). In humans prenatally exposed to famine, but exposed to an abundance of food later in life, this has led to higher rates of obesity (Roseboom et al., 2006) and diabetes (Kahn et al., 2009). Furthermore, differentially methylated regions were found in whole blood when compared to their siblings and these regions were associated to prenatal malnutrition, early development, metabolism and growth (Tobi et al., 2009, 2014). Thus, the consequences of early life conditions might be mediated by adjusting metabolic efficiency and DNA methylation might be one of the mechanisms behind this.

One gene, *CIQC*, is a regulator of the immune response and synapse development and was previously associated to cognition and behavior. *CIQC* expression has been associated to Alzheimer's disease and alterations in learning behavior (Khoonsari et al., 2016; Haure-Mirande et al., 2019), ADHD and autism (Corbett et al., 2007; Trent et al., 2014). However, the direction of the effect is not completely understood and might be dependent on the developmental stage (Davies et al., 2009; Trent et al., 2014). The DMS in *CIQC*-like was hypermethylated in the reduced pool compared to the enlarged pool, suggesting lower *CIQC*-like expression in nestlings from the reduced broods. Although the role of *CIQC* in development of Alzheimer's disease, ADHD and autism is not fully understood yet, this might indicate a difference in brain development, synaptic structure, behavior and cognition between the two treatment groups. This is expected based on previously found effects of food availability, diet quality and brood size on behavior (Carere et al., 2005; Krause et al., 2009; van Oers et al., 2015) and cognition (Nettle et al., 2015) in birds. Food availability is known to affect nestling stress response (van Oers et al., 2015) and exploratory behavior (Carere et al., 2005) and diet quality affects the latency to approach food and feed later in life (Krause et al., 2009). Furthermore, small natal brood size has been associated

to slow conditioning to a stimulus and slow reversal learning (Nettle et al., 2015).

The genes and their functions described above are supported by the significantly enriched GO terms developmental process, regulation of multicellular organismal process, anatomical structure morphogenesis, anatomical structure development and regulation of cell differentiation, which all indicate a difference in development between nestlings from enlarged and reduced broods. Although more GO terms were significantly enriched in the comparison of the two control pools and these were similar to those in the comparison of the two treatment pools, more GO terms in the comparison of the treatment pools were highly significantly enriched. This means that more GO terms were highly enriched when comparing reduced with enlarged nestlings than when comparing nestlings from control broods.

Overall, we show that experimental brood size variation leads to more differential DNA methylation in more regulatory regions of genes than when performing a control experiment. This indicates that DNA methylation in response to experimental variation in brood size has the potential to alter gene expression. Most of the genes were functional in tissues other than blood and this may affect how gene expression is related to a trait, due to tissue differentiation. However, multiple studies have shown that gene expression levels in the blood were related to the processes associated with that gene (Roulin et al., 2011; Zhu et al., 2017; Lindner et al., 2021b), suggesting that gene expression levels in blood in our study could explain plasticity in phenotypic traits related to brood size variation. Although these results demonstrate that early life stress affects epigenetic regulation of genes related to brood size, namely genes that are known to affect development, growth, metabolism, behavior and cognition, future work is needed. Further work should assess the causal effects of changes in DNA methylation on gene expression at these loci and related phenotypic traits, and specifically to find repeatable results in similar experiments. Furthermore, it should be assessed whether a single DMS can affect gene expression.

The expectation was that we would not find any differentially methylated sites when comparing pools of control siblings raised in different broods. Still, we found several DMS in the regulatory regions of the genes *HSPA2*, *METTL8*, *ZSWIM1*-like (*LOC107213098*) and *OSMR*-like (*LOC107198170*). These results show that our control pools were not identical in terms of DNA methylation or that these results are false positives. However, we expect the chance of these DMS to be false positives to be very slim because of our stringent approach during data analysis; we applied coverage filtering, a threshold of 25% differential methylation and a Bonferroni corrected α -threshold. Furthermore, we cannot link these differences to our experimental approach. A previous study suggested an effect of manipulation (i.e., being moved to a different nest) on DNA methylation in zebra finches (Sheldon et al., 2018), which was controlled for in the current experimental approach. This means that this cannot explain the DMS and the direction of methylation in the control comparison. One likely explanation may be the existence of large individual variation in DNA methylation (Viitaniemi et al., 2019). By pooling individuals we tried to focus on average group DNA methylation

(Docherty et al., 2009) instead of individual variation. However, the number of individuals in our pools might have been too small to completely discard such individual differences. This suggests that some of our DMS in the treatment comparisons might have also been caused by individual differences. Nevertheless, PCA revealed that the reduced pool and the enlarged pool clustered together very closely, which indicates that our experimental setup, matching sibling pairs that were raised in differently sized broods, worked, since these pools were very comparable.

The fact that our control pools were not as similar as thought was supported by the finding that the hyper/hypo distribution between the control broods is non-equally divided with all differentially methylated sites being hypermethylated in Control1. This indicates that by chance some factor that induces DNA methylation in a certain direction was present in one pool. Furthermore, the two control pools did not perfectly cluster together in the PCA plot, unlike the reduced and enlarged pools. This is surprising, since we would expect control pools to cluster in the same way, since siblings were paired there as well. We therefore conclude that the controls pools were not balanced, causing the number of DMS to be higher than if the control pools would have been balanced. One reason could be a biased sex ratio (Natt et al., 2014). However, only one DMS in a regulatory region was situated on the sex chromosome, which was equal to the number found in the treatment comparison. One DMS was found in *HSPA2*, a gene involved in protein refolding and in spermatogenesis. Given the importance of this gene for male fertility (Dix et al., 1996; Son et al., 1999), this might indicate a difference in expression between the pools, which might be caused by a biased sex ratio in the individuals included in the pools, although this methylation change does not have to be sex dependent. In great tits, visual determination of the sex before the first molt is unreliable, and most birds from this study were not recaptured after the first molt and have therefore not been molecularly sexed, making it impossible to balance the number of males and females in the pools. Since a DMS in *HSPA2* might also indicate a bias in brood temperature, because *HSPA2* expression is affected by temperature in chicken testes (Wang et al., 2013, 2015), we do not expect our main findings to be affected by a potential sex-bias. Another possible, but unlikely explanation could be that the differences were caused by genetic variation between the pools, since a large fraction of erythrocyte DNA methylation is similar between relatives (Viitaniemi et al., 2019; van Oers et al., 2020). However, since the samples in one pool were, to our knowledge, unrelated and the samples in Control1 originated from siblings of the samples in Control2, we expect the genetic diversity to be larger within than between the pools, although we did not check for extra-pair paternity, which is estimated at about 10% for this population (van Oers et al., 2008). Therefore, it could be that nestlings of one sibling pair were half-siblings, because they were sired by different males. However, since we do not have individual DNA methylation information, we can only speculate on these causes and functional validation of the candidate loci is needed to assess the causal relationship between our experiment and the change in methylation, highlighting the need for studies that assess individual variation in DNA methylation levels.

Although, with our design, we cannot disentangle these possible alternative explanations explaining the high number of DMS and the direction of methylation in the control comparison. However, since the two treatment pools were, unlike the two control pools, very similar, we do think that the difference between the number of DMS in the control comparison and the treatment comparison is conservative rather than exaggerated. Therefore, we conclude that most of the DMS in the treatment comparison are likely related to the treatment and the DMS between the control pools may also be caused by some non-explained bias in the control pools.

In conclusion, to our knowledge, this is the first study that investigates the effects of experimentally altered brood size on genome-wide DNA methylation in a wild bird population and that disentangles prehatching from rearing effects with a partial cross-foster design, controls for possible effects of manipulation and assesses the functionality of annotated differentially methylated sites. Our work demonstrates that early life stress due to variation in brood size directly affects epigenetic regulation of genes that are known to affect brood-size dependent phenotypes, such as development, growth, metabolism, behavior and cognition. Although future studies are needed to validate our findings, this study underlines the potential role for DNA methylation in the intergenerational regulation of developmental phenotypic plasticity in altricial birds.

DATA AVAILABILITY STATEMENT

The datasets presented in this study can be found in online repositories. The names of the repository/repositories and accession number(s) can be found below: <https://www.ncbi.nlm.nih.gov/biosample/14093678>; <https://www.ncbi.nlm.nih.gov/biosample/14093679>; <https://www.ncbi.nlm.nih.gov/biosample/14093680>; and <https://www.ncbi.nlm.nih.gov/biosample/14093681>.

ETHICS STATEMENT

The animal study was reviewed and approved by the Institutional Animal Care and Use Committee (NIOO-IvD) and the

Koninklijke Nederlandse Akademie van Wetenschappen – Dier Experimenten Commissie (KNAW-DEC licenses NIOO 10.07 and 14.11 to KO).

AUTHOR CONTRIBUTIONS

BS and KO designed the study and obtained funding. BS conducted the experiment. BS and JE conducted the data analysis with help of FG and VL. BS, JE, and KO drafted the manuscript. KO supervised the study. All authors contributed to editing the manuscript.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: <https://www.frontiersin.org/articles/10.3389/fevo.2021.609061/full#supplementary-material>

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Conflict of Interest: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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